DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR
ACETOBACTER ACETI

I. SUMMARY

Acetobacter aceti is a benign microorganism that is ubiquitous in the environment existing in ecological niches such as flowers, fruits, honey bees, as well as in water and soil. It has a long history of safe use in the fermentation industry for the production of acetic acid from alcohol. There are no reports in the literature suggesting that A. aceti is a pathogen of humans or animals. It is not considered a plant pathogen, but there are reports of A. aceti causing pink disease of pineapple and rot of apples and pears. However, the taxonomy of the genus has been revised since these reports were published, and it is doubtful whether the microorganism that is presently classified as A. aceti was actually responsible for either of these diseases. A. liquefaciens (formerly classified as A. aceti subsp. liquefaciens) is the microorganism that causes pink disease of pineapple which results in brown discoloration of fruit during processing. It appears that strains of A. aceti may be capable of causing rot in apples, but only with mechanical injection of high concentrations of bacteria below the outer protective epidermal layers.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material

meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emissions specify that liquid and solid waste containing the microorganisms be

treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed

individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for the Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the

introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Acetobacter aceti, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of A. aceti will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of A. aceti, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of A. aceti, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place

the microorganism on the list. The Agency's specific determination for Acetobacter aceti is discussed in the next unit.

III. EVALUATION OF THE CANDIDATE

A. History of Use

1. History of safe commercial use. Members of the genus Acetobacter have been used industrially since the 1850s, primarily for food grade acetic acid (vinegar) production. A. aceti is considered a Class 1 Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and is included on the FDA's GRAS list of microorganisms.

2. Products subject to TSCA jurisdiction. EPA has not yet received a submission for use of A. aceti under TSCA. However, potential TSCA uses of acetic acid include manufacturing of acetate rayon, plastics production, rubber production, and photographic chemicals.

B. Identification of Microorganism

1. Classification of microorganism. The genus Acetobacter is well-defined. It has recently been reclassified following a numerical analysis of 177 phenotypic characteristics of organisms in the genus. There are several differentiating characteristics which enable identification of the individual species of Acetobacter. Because the genus has recently been revised, older strains in use for acetic acid production may not be correctly classified as A. aceti.

2. Related taxa of concern. Acetobacter liquefaciens, formerly classified as A. aceti subsp. liquefaciens, has been associated with pink disease of pineapple, through its production of 2,5-diketogluconic acid which causes tissue to turn pink. The pink tissue then turns brown after heat treatment during processing. A. liquefaciens may also be involved in rot of apples and pears, which in some cases has been associated with 2,5-diketogluconic acid production. A. aceti does not produce 2,5-diketogluconic acid.

C. Risk Summary

1. Studies regarding potential for adverse effects. There are no reports in the literature suggesting that A. aceti is pathogenic to humans or animals. A. aceti does not produce toxins, enzymes, or other extracellular virulence factors

normally associated with pathogenicity. While A. aceti has been reported as the causal agent of pink disease of pineapple, these reports were published prior to the reclassification of the genus Acetobacter. The organism now classified as A. liquefaciens is considered to be responsible for pink disease of pineapple through its production of 2,5-diketogluconic acid, an acid which is not produced by A. aceti. It appears that strains of A. aceti may be capable of causing rot in apples, but only with mechanical injection of high concentrations of bacteria below the outer protective epidermal layers.

2. Studies regarding survival in the environment. A. aceti is ubiquitous in the environment, existing in ecological niches such as flowers, fruits, honey bees, as well as in water and soil. It is found essentially wherever sugar fermentation occurs providing ethanol as a substrate for conversion to acetic acid.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. A. aceti is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. In addition to assessing the risk of A. aceti, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. RECOMMENDATION: Acetobacter aceti is recommended for the TSCA section 5(h)(4) tiered exemption.

B. RATIONALE

1. Risks from use of the recipient microorganism A. aceti are low. A. aceti is a benign microorganism which is included in the FDA's GRAS list. It is not pathogenic to humans or animals. Although it often comes in contact with humans due to its widespread presence in the environment, it does not colonize human skin nor does it inhabit the human body. There are no reports in the literature suggesting any allergic or immunologic responses to the bacterium that has been used for decades in fermentation facilities. Releases of this microorganism to the environment through fermentation uses would not pose any significant ecological hazards, because this microorganism is ubiquitous in the environment and it is not pathogenic to animals or plants.

2. Use of strains of A. aceti which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. A. aceti is considered to be a benign organism. Reports of pink disease of pineapples and rot in pears and apples are considered to be primarily attributable to A. liquefaciens which was formerly classified as A. aceti. Because of the change in classification, older industrial strains of A. aceti may not meet the present-day designation of A. aceti. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using A. aceti. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

Attachment 1:

INTEGRATED RISK ASSESSMENT OF
ACETOBACTER ACETI

I. INTRODUCTION

Acetobacter aceti is a benign microorganism that is ubiquitous in the environment, existing in alcoholic ecological niches such as flowers, fruits, honey bees, as well as in water and soil. It has a long history of safe use in the fermentation industry for the production of acetic acid from alcohol. There are no reports in the literature suggesting that *A. aceti* is a pathogen of humans or animals. It also is not considered a plant pathogen. The potential risks to human health or the environment associated with the use of this bacterium in fermentation facilities are low. Since the taxonomy of the genus was recently revised, some older production strains in use for acetic acid production may, in fact, not meet the current taxonomic designation of *A. aceti*.

History of Commercial Use & Products Subject to TSCA Jurisdiction

The history of safe use for this bacterium is predominately for food grade acetic acid (vinegar) production. Members of the genus *Acetobacter* have been used industrially since the 1850's (Edberg, 1991). *A. aceti* has also been reported in the literature as being used for cellulose production for specialty papers or headphones (Anonymous, 1989a, 1989b); however, strains capable of cellulose production are classified as *A. pasteurianus* or *A. hansenii* under the new taxonomic system (De Ley et al., 1984). *A. aceti* is considered a Class 1 Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986), and is on the FDA's GRAS (generally recognized as safe) list of microorganisms.

There are a number of TSCA applications for acetic acid. These include manufacturing of acetate rayon, plastics production, rubber production, and photographic chemicals.

II. IDENTIFICATION AND TAXONOMY

A. Overview

Acetobacter aceti is a Gram negative bacterium which is motile by peritrichous flagella. It is obligately aerobic

possessing only the ability for respiratory metabolism with no fermentative ability. *A. aceti* does not form endospores. This bacterium is ubiquitous in the environment, existing in soil, water, flowers, fruits, and on honey bees; in essence, wherever sugar fermentation is occurring. *A. aceti* produces acetic acid from ethanol in alcoholic niches in the environment. Acetate and lactate are oxidized to CO_2 and H_2O by the organism. The optimal temperature for growth is between 25 to 30C, and the pH optimum between 5.4 to 6.3 (De Ley et al., 1984). *A. aceti* is a common contaminant in all industrial fermentation facilities and is responsible for generating turbidity, ropiness, discoloration, and off-flavors in beer (Kough, 1991).

B. Taxonomy and Characterization

The genus *Acetobacter* is well-defined, although changes in the taxonomy have occurred in recent years. In the 1974 edition of Bergey's Manual of Determinative Bacteriology, there were three species in the genus: (1) *A. aceti* with four subspecies (*aceti, orleanensis, xylinum*, and *liquefaciens*); (2) *A. pasteurianus* with five subspecies (*pasteurianus, lovaniensis, estunensis, ascendens*, and *paradoxus*); and (3) *A. peroxydans*. All these species and subspecies appear on the Approved Lists of Bacterial Names of 1980.

However, the most recent edition of Bergey's Manual of Systematic Bacteriology (De Ley et al., 1984) has reclassified the genus after a numerical analysis was conducted on 177 phenotypic characteristics of organisms in the genus. Presently the genus consists of four species: *A. aceti, A. liquefaciens* (formerly *A. aceti* subsp. *liquefaciens*), *A. pasteurianus* (formerly *A. aceti* subsp. *xylinum* and *orleanensis*), and *A. hansenii*. There are several differentiating characteristics which enable identification of the individual species of *Acetobacter*.

It is important to note that since the genus has recently been revised, older strains in use for acetic acid production may not be correctly classified as *A. aceti*. This risk assessment is for the present-day designation of *A. aceti*.

C. Related Species of Concern

A. liquefaciens, although not considered to be a plant pathogen, has been reported to cause problems in stored fruit. This bacterium does not appear on a Department of Agriculture list of plant pathogens (USDA, 1988), nor is a USDA permit required to have the bacterium in one's possession (Kough, 1991). However, *A. liquefaciens* is capable of producing 2,5-

diketogluconic acid which causes pink disease of pineapple (De Ley et al., 1984). This disease is characterized by the discoloration of the tissue to pink which then turns brown with heat during processing (De Ley et al., 1984). Apparently, the fruit itself is unaffected and the browning during processing can be avoided if the fruit is washed prior to processing (Kough, 1991). *A. liquefaciens* may also be involved in rot of apples and pears which has been shown, in some cases, to be associated with the production of 2,5-diketogluconic acid (Van Keer et al., 1981).

III. HAZARD ASSESSMENT

A. Human Health Hazards

A. aceti has not been reported as a human pathogen. It is ubiquitous in the environment, and therefore, comes in contact with humans on a frequent basis. Its optimum growth temperature is below that of the human body and its optimum pH is below that normally found on the surface of human skin. Due to its close association with sugar breakdown, it is unlikely that this species would form part of the normal bacterial flora of humans (Edberg, 1992). Review articles on the normal flora of the human body did not reveal *A. aceti* (Edberg, 1992).

There are no reports in the literature that *A. aceti* is capable of producing toxins active against humans or animals, nor are there reports of *A. aceti* causing infection in humans or animals (Edberg, 1992). It does not produce enzymes or other extracellular factors normally associated with virulence. There is no reason to suspect that *A. aceti* could acquire or transfer any virulence factors. This bacterium does possess plasmids which are responsible for the production of enzymes used in acetic acid production. These plasmids have been shown to be transferred to other members of the species in the laboratory under optimal conditions. However, there is no evidence of plasmid transfer between strains of *A. aceti* or related species in the environment. Its unique ecological niches are such that it is unlikely that a second recipient or donor microorganism would be present in quantities sufficient for plasmid exchange to occur (Edberg, 1992).

Biochemical characteristics of *A. aceti* virtually preclude it as being a threat to human health. Although it grows well with ethanol as a source of carbon, glucose has been shown to actually decrease the growth rate in culture, especially when other carbon sources were present (O'Sullivan and Ettlinger, 1976). In addition, industrial strains may have been selected so

that they do not have the ability to grow on glucose (Weber and Ettlinger, 1971) or so that they utilize very specific amino acids as nitrogen sources. This may result in growth inhibition in the presence of alternate amino acids (O'Sullivan, 1974).

In summary, *A. aceti* has no demonstrated virulence factors. It is not part of the normal flora of human skin or the body and is not expected to survive in a human host for sustained periods of time. The only threat to human health would lie in a massive contamination event in which workers may be exposed to extraordinarily high concentrations of the bacterium, and perhaps, develop an allergic or immunological reaction. It appears, however, because the bacterium is used for acetic acid production, should such a contamination event occur, the acetic acid would present a greater threat to workers than the bacterium itself. The potential for human virulence is virtually nonexistent for *A. aceti* (Edberg, 1992).

B. Environmental Hazards

1. Hazards to animals

There are no reports in the literature suggesting that *A. aceti* is pathogenic to animals. As previously mentioned, the bacterium does not produce toxins, enzymes, or other extracellular virulence factors normally associated with pathogenicity (Edberg, 1992).

2. Hazards to plants

A. aceti has been reported as being the causal agent of pink disease of pineapple (Kontaxis and Hayward, 1978; Cho et al., 1980). This disease is characterized by a pink discoloration of the fruit which turns brown with heat during processing. The production of the metabolite 2,5-diketogluconic acid is responsible for the discoloration associated with pink disease of pineapple.

These reports suggesting that *A. aceti* is the cause of pink disease of pineapple were published prior to the reclassification of the genus *Acetobacter*. *A. aceti* does not produce 2,5-diketogluconic acid. It is the bacterium now designated as *Acetobacter liquefaciens* (formerly *A. aceti* subsp. *liquefaciens*) which is responsible for this disease through the production of 2,5-diketogluconic acid, not *A. aceti*.

There is another report in the literature of a disease of stored fruit presumably caused by *A. aceti*. This organism, as well as numerous acetic acid bacteria and other bacteria, were

reported to cause rot in apples and pears resulting in different degrees of browning (Van Keer et al., 1981). This study involved inoculating apples and pears with 172 strains of bacteria including a variety of acetic acid bacteria from the genera *Acetobacter, Gluconobacter*, and other genera. The entire fruits were inoculated by either 10 mm-deep stab wounds with inoculating needles or by injection to a depth of 10 mm of 0.2 ml of the same pure culture of bacteria with a density of 10^8 cells/ml. Alternatively, sections of the epidermis were removed from the fruit and the bacteria were swabbed over the exposed tissue. Fruits were incubated for two weeks in sterile plastic bags. In other experiments, 4 to 6 mm discs obtained from surface-disinfected fruit were inoculated with the bacterial suspensions by an infected inoculation needle and incubated in petri dishes.

All of the inoculation methods except stab inoculation resulted in rot of apple tissue with most of the acetic acid bacteria tested, including seven strains of what was formerly designated as *A. aceti* subsp. *aceti* and nine strains of other subspecies of *A. aceti*. With the 15 varieties of apples tested, it was concluded that the surface of the fruit must be wounded in order to obtain rot. The three pear varieties tested were shown to be more susceptible to rot, and wounding of the surface was not necessary.

The ability to cause rot of apples and pears as suggested in the above paper may be questionable for bacteria presently designated as *A. aceti*. First, this article was written before the revision of the genus *Acetobacter*, therefore, it is difficult to tell if the strains used would meet the current designation of *A. aceti*.

Second, in order to satisfy Koch's postulates, re-isolations of pure cultures were made from the rotting fruit that had been injected with one strain of *Acetobacter* (species not specified), and two strains of *Gluconobacter* (species not specified). In all cases, 2,5-diketogluconic acid and 2-ketogluconic acid were isolated from the fruit. *A. aceti* can produce 2-ketogluconic acid and 5-ketogluconic acid but does not produce 2,5-diketogluconic acid. The disease of apples and pears is thought to be biochemically similar to pink disease of pineapple (Edberg, 1992). As previously mentioned, pink disease of pineapple is caused by the production of 2,5-diketogluconic acid, which is produced only by *A. liquefaciens*, not *A. aceti* or other species of *Acetobacter*.

Third, although rotting symptoms appeared in fruits that were mechanically inoculated with high concentrations of

acetic acid bacteria, rotting symptoms were also demonstrated with 32 strains of bacteria from various genera, some of which are known pathogenic bacteria, but others have no association with plant pathogenicity. The additional bacteria studied included strains from the following genera: *Xanthomonas, Pseudomonas, Frateuria, Escherichia, Agrobacterium, Alcaligenes, Erwinia, Serratia, Paracoccus, Klebsiella, Proteus, Flavobacterium*, and *Chromobacterium*. According to the U.S. Department of Agriculture regulations on biotechnology products under the Federal Plant Pest Act (7 CFR 330, et seq.), no members of the genera *Fratueria, Escherichia, Alcaligenes, Serratia, Parococcus, Klebsiella, Proteus, Flavobacterium*, or *Chromobacterium* are considered plant pathogens, nor are any species of these genera suggested as being plant pathogens according to Bergey's Manual of Systematic Bacteriology (De Ley et al., 1984). Consequently, it appears as though a variety of nonpathogenic bacteria are capable of producing rot symptoms in pears and also in apples when inoculated at high concentrations into the soft tissue below the outer protective epidermal layers.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

A. aceti is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986). This bacterium also falls under the Class 1 Containment (harmless microorganism) under the European Federation of Biotechnology guidelines (Frommer et al., 1989).

No data were available for assessing the release and survival specifically for fermentation facilities using *A. aceti*. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and

monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Releases

Estimates of the number of *A. aceti* organisms released per production batch are presented in Table 1. The minimally controlled scenario assumes no treatment of fermentor off-gas and a 2-log reduction in cell density of the fermentation broth after inactivation. The full exemption scenario assumes the use of in-line filters for 99% removal of microorganisms from fermentor off-gas and a 6-log reduction in cell density of the fermentation broth after inactivation (Reilly, 1991).

TABLE 1. Estimated Number of Viable A. aceti Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$2x10^{6}$ - $1x10^{9}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{13}$	$7x10^{9}$	90
Soil/Landfill	$7x10^{15}$	$7x10^{11}$	90

Source: Reilly, 1991

These release figures assume a batch production process. However, acetic acid production is also accomplished using a semi-continuous process (De Ley et al., 1984). Releases are expected to be lower under a semi-continuous process (LaVeck, 1991).

2. Air

Organisms released to air will most likely decrease in number due to UV light, temperature, desiccation, and a lack of nutrients. Those that do remain viable and slowly drift to land may be capable of establishing themselves in soil, although these numbers are probably negligible. Human exposure is expected to be low since the numbers of organisms released would quickly be diluted in the atmosphere and some die-off of the organisms would occur (LaVeck, 1991).

3. Water

Surface water concentrations of microorganisms were estimated using the 10% and 50% flow values for SIC code 283 facilities (drugs, medicinal chemicals, pharmaceuticals) that release to surface water. The SIC code flow was estimated using 128 indirect (facilities that send their waste to a POTW) and direct dischargers (facilities that have a NPDES permit to discharge to surface water). Discharger data were extracted from the IFD (Industrial Facilities Dischargers) database and surface water flow data were taken from the RXGAGE database, maintained by the EPA. These data were partitioned into percentile rankings and flows for the 10th percentile (small river) and 50th (average river) were extracted and used for the exposure calculations. Flow is expressed in millions of liters/day (MLD). Mean Flow is

the average flow value, and 7Q10 flow is the lowest flow observed over 7 consecutive days during a 10 year period. Concentrations of microorganisms in surface water are calculated for both the minimally controlled and the full exemption scenarios. The surface water concentrations assume 100% survival of the organisms in the treatment plant (LaVeck, 1991). Estimated concentrations of *A. aceti* in surface water for the minimally controlled and full exemption scenarios are presented in Table 2.

TABLE 2. A. aceti Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	159	4.57	4.4×10^{5}	1.53×10^{7}
50th Percentile	768	68.13	9.11×10^{4}	1.03×10^{7}
Full Exemption				
10th Percentile	159	4.57	4.4×10^{1}	1.53×10^{3}
50th Percentile	768	68.13	9.11×10^{0}	1.03×10^{2}

*MLD = million liters per day
Source: LaVeck, 1991

These numbers represent a worst-case, because they are based on 100% survival in the treatment plant which is unlikely due to pH, desiccation, predation, and competition for nutrients.

4. Soil

Since *Acetobacter* is a common soil inhabitant, survival in soil would be expected. However, the method of disposal would influence survival. For example, landfilling the organisms would probably not result in long term survival since the anaerobic conditions in landfills would result in cell death. If the organisms were spread out over the surface of the soil, then aerobic conditions would prevail. However, on the soil surface, the bacteria are subject to die-off from UV, temperature, and desiccation. Once in the soil, *A. aceti* populations are expected to decline and reach a steady state population. These releases could result in some human and environmental exposure (LaVeck, 1991).

V. INTEGRATION OF RISK

A. Discussion

Acetobacter aceti is ubiquitous in the environment occupying alcoholic niches such as flowers, fruits, honeybees, as well as soil and water. It is found essentially wherever sugar fermentation occurs providing ethanol as a substrate for conversion to acetic acid.

The actual history of the use of *A. aceti* for production of acetic acid from ethanol is not known, however, members of the genus have been used industrially since the 1850's (Edberg, 1992). The main industrial use of *A. aceti* is for the production of vinegar which is not a TSCA application. However, there are TSCA applications for acetic acid including the manufacture of rubber, plastics, acetate fibers, and photographic chemicals.

A. aceti is a benign microorganism. It is included in the Food and Drug Administration's GRAS (generally recognized as safe) list (CFR 21, Parts 170-179, April 1, 1988). It is not pathogenic to humans. Although it often comes in contact with humans due to its widespread presence in the environment, it does not colonize human skin, nor does it inhabit the human body. There are no reports in the literature suggesting any allergic or immunological responses to the bacterium that has been used for decades in fermentation facilities. *A. aceti* does not produce any toxins, enzymes, or virulence factors that usually are associated with pathogenicity. In addition, certain biochemical characteristics of the bacterium such as decreased growth on glucose and growth inhibition in the presence of certain amino acids also lessen the likelihood of human pathogenicity. The potential for human virulence is virtually nonexistent for this species (Edberg, 1992). In addition, worker exposure to the organism is expected to be low (Reilly, 1991).

A. aceti is expected to survive in the environment if released from the fermentation facility. However, exposure to the environment through exhaust gases or liquid wastes are expected to be low under the conditions for inactivation required for this exemption (LaVeck, 1991). Any releases which would occur would not pose any significant ecological hazards, as this microorganism is already ubiquitous in the environment and it is not pathogenic to animals or plants (Kough, 1991). In older literature, prior to the revision of the genus in 1984 (De Ley et al., 1984), *A. aceti* was reported to cause pink disease of pineapple and rot in pears and apples. The former disease is caused by *A. liquefaciens* which was formerly classified as *A.*

aceti. Doubt exists on the species identity with the report on rot in apples and pears, since a certain metabolite was found in all infected fruits (Van Keer et al., 1981) but *A. aceti* cannot produce that metabolite (De Ley et al, 1984). In any case, *A. aceti* does not result in typical disease symptoms such as decreased growth or yield loss, and therefore, cannot be considered a true pathogen. In addition, many other nonpathogenic bacteria were also found to cause rot in pears or in apples when mechanically inoculated beneath the surface of the outer protective epidermal layers. Rot of apples and pears caused by *A. aceti* must not occur frequently in nature, as this is the only citation in the literature reporting this problem. In summary, even if *A. aceti* is released to the environment and is capable of producing rot in pears or rots in apples with damaged epidermal layers, the exposures to orchards or fruit processing plants will probably not be great, or at least not substantially greater than the exposure from strains of *A. aceti* ubiquitous in the environment.

The only point of concern regarding this microorganism is in regards to the taxonomic revision of the genus *Acetobacter* in the early 1980's. Presently, *A. aceti* is well-defined as a species and is readily distinguished from other *Acetobacter* species. However, older industrial strains of *A. aceti* may not, in fact, meet the current designation of *A. aceti*. Since there are some potential hazards associated with related species in this genus, industrial strains should be verified as being correctly identified as *A. aceti* using the revised taxonomic classification scheme.

B. Recommendation

All strains of *Acetobacter aceti* are recommended for the tiered exemption provided that these strains meet the current taxonomic definition of *A. aceti*.

VI. REFERENCES

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